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ORIGINAL PAPER

Electrochemical aptasensor for tetracycline detection

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Abstract An electrochemical aptasensor was developed for the detection of tetracycline using ssDNA aptamer that selectively binds to tetracycline as recognition element. The aptamer was highly selective for tetracycline which distinguishes minor structural changes on other tetracycline derivatives. The biotinylated ssDNA aptamer was immobilized on a streptavidin-modified screen-printed gold electrode, and the binding of tetracycline to aptamer was analyzed by cyclic voltammetry and square wave voltammetry. Our results showed that the minimum detection limit of this sensor was 10 nM to micromolar range. The aptasensor showed high selectivity for tetracycline over the other structurally related tetracycline derivatives (oxytetracycline and doxycycline) in a mixture. The aptasensor developed in this study can potentially be used for detection of tetracycline in pharmaceutical preparations, contaminated food products, and drinking water.

Keywords Electrochemical detection · Aptasensor · DNA aptamers · Tetracycline

Introduction

Tetracycline is an antibiotic which reduces affinity for prokaryotic tRNA by strong binding on the 30S ribosomal subunit [1, 2]. This antibiotic molecule has been used for animals as growth promoters. Residual tetracycline group has been detected in various organs and muscles of slaughtered animals [3]. Tetracyclines are also found in food products, such as meat [4, 5], milk [6], honey [7], and chicken's egg [8]. Also, tetracycline is known to be hepatotoxic agent, especially for pregnant women [9] which leads to a serious threat to human health. Therefore, many researchers are trying to develop an analysis tool for detection of tetracycline antibiotics (TCs) in food products, pharmaceutical preparations, and water. The most common and classical techniques that have been used so far are HPLC [10, 11], capillary electrophoresis [12], and chemiluminescence [13]. However, these detection methods for TCs are often time-consuming and expensive that lack specificity and always need the authentic samples as reference standards.

Several other analysis methods for detection of TCs in serum, meat and milk samples have been reported, such as dipstick colorimetric method [14]. However, this method is neither sensitive for detection of TCs nor distinguishes the other members of TET derivatives in contaminants. In another study, an improved amperometric detection method for TCs was reported by using multi-wall carbon nanotube-modified electrodes (detection limit: 0.09–0.44 μM of oxytetracycline) [15]. Immune-based colorimetric methods employing ELISA-type format which utilize antibodies had the detection limit of TCs in ng/mL range [16], but these sensitive detection techniques for TCs are often expensive, labor intensive, and time-consuming.

Recently, aptamer-based biosensors have been studied extensively because of their several advantages using aptamers as sensing probes. This is because the aptamers have high affinity and specificity to their target molecules; they are easy to modify and labeling, smaller in size, no limitation against any types of targets, reversible denaturation, and have high thermal stability. Based-on these advantages, numerous studies have been reported on

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aptasensors utilizing different signal transducers, such as optical [17–19], mass-dependant [20–22], colorimetric [23–25], and more recently on electrochemical systems [26, 27]. Electrochemical analysis is an attractive platform because it is simple, rapid, and also cost-effective. Therefore, a large number of studies on development of electrochemical aptasensors have been reported. Various strategies have been adapted for signal generation, amplification, and development of novel and sensitive electrochemical aptasensors, particularly for protein targets have been reported [28–31]. The other methods include nanoparticle-based aptasensors for signal amplification [32–34], signal-on measurement by the conformational change of aptamers [35–37], and a target-induced displacement method [38–41].

There are limited number of studies on aptasensors particularly for the detection of small organic molecules and no report particularly on detection of tetracycline. In this study, therefore, we have developed a simple electrochemical aptasensor for the specific detection of tetracycline using tetracycline binding ssDNA aptamer which was developed in our previous study [42]. This aptamer was highly specific and selective to TET and was immobilized on screen-printed gold electrode via avidin–biotin interaction, and the binding of TET to aptamer was analyzed by cyclic voltammetry (CV) and square wave voltammetry (SWV).

Materials and methods

Apparatus

Electrochemical analysis was performed at room temperature using an electrochemical analyzer PGSTAT30 Autolab (Ecochemi, Utrecht, The Netherlands). The screen-printed gold electrodes composed working, reference, and counter electrode all of which were integrated, and the working electrode had a diameter of 1.6 mm (DropSens, S.L., Spain).

DNA aptamer and reagents

A TET binding ssDNA aptamer was first modified by extended with a poly-T tail (T_5) for flexibility, and spacing at the 5′ terminal and labeled with biotin to immobilize on streptavidin-modified gold electrode. The dissociation constant (K_d) of TET binding DNA aptamer was calculated as 63.6 nM in our previous study [42]. The sequence of TET binding-modified ssDNA aptamer is as follows: 5′-Biotin-TTTTTCGTACGGAATTCGCTA GCCCCCCG GCAGGCCACGGCTTGGGTTGGTCCCACTGCGCGTG GATCCGAGCTCCACGTG-3′. The secondary structure of TET binding ssDNA aptamer was predicted by Mfold program according to the free-energy minimization algorithm (Fig. 1) [43].

The aptamer was chemically synthesized and purchased from Genotech Co. (Daejeon, Korea). Tetracycline, doxycycline, oxytetracycline, diclofenac, potassium hexacyanoferrate (II) trihydrate, 3,3′-dithiodipropionic acid, and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) were from Sigma Co. (USA). The *N*-hydroxysuccinimide (NHS) and streptavidin were purchased from Fluka (USA).

Preparation of tetracycline aptamer-modified gold electrodes

The working (gold), reference (silver), and counter (gold) electrodes are integrated on a single chip, and the diameter of working electrode was 1.6 mm (Fig. 2a). The electrodes were washed with 10 mM of H_2SO_4 under electric potential within –0.2 to 1.6 V and monitored by cyclic voltammetry (CV). To confirm the CV curve, the three electrodes were etched with 0.5 mM of potassium hexacyanoferrate (II) trihydrated (ferrocyanide, $K_3Fe(CN)_6$) solution containing 10 mM KCl (Sigma Co., USA) under electric potential within –0.2 to 0.6 V.

After the etching step, the working electrode was treated with 200 mM of 3,3′-dithiodipropionic acid for 30 min to form a self assembled monolayer (SAM), and then washed thoroughly with distilled water. The activation of carboxylic groups were performed on the electrode after incubation with 100 mM of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) and 1 mM *N*-hydroxysuccinimide (NHS) for an hour. Afterward, the electrode was incubated overnight with

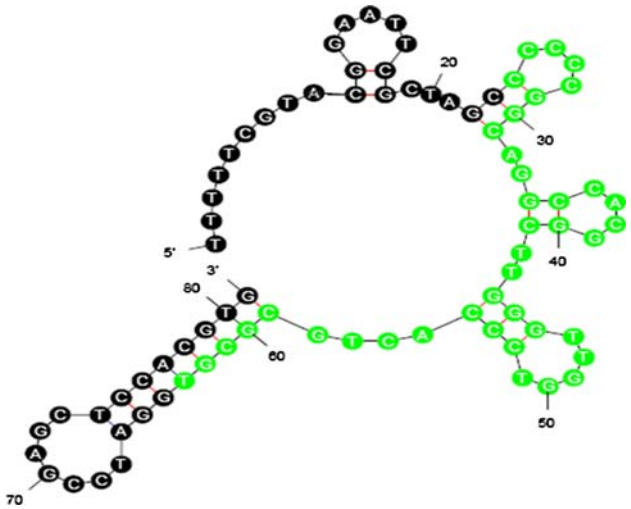

Fig. 1 Secondary structure of a biotin-labeled and poly-T_5 tagged TET binding aptamer (modified TET binding aptamer) predicted by Mfold program. Variable N_{40}-nuceotide region in the modified TET binding aptamer is highlighted in *green* (24th–63rd nucleotides). The *inset* shows original TET binding aptamer (No. 20)

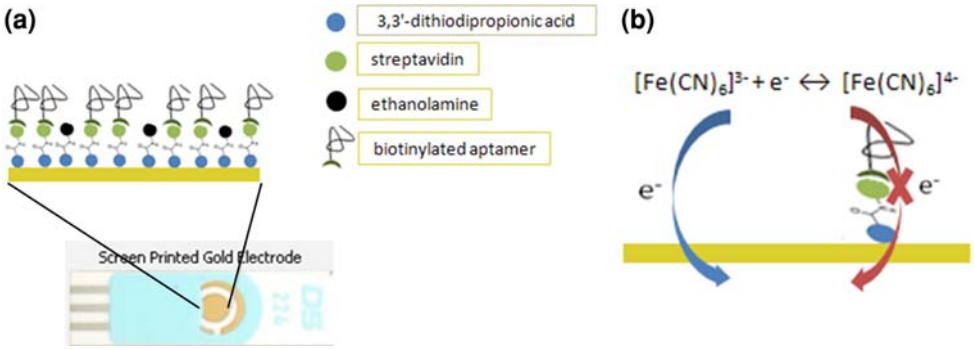

Fig. 2 A schematic diagram of **a** ssDNA aptamer immobilized on the working electrode region of a screen-printed electrode chip. **b** Scheme of signal off system through interference in the electron transfer

1 mg/ml streptavidin in PBS buffer (pH 7.5). The free carboxyl groups on the electrode were blocked by incubation with 100 mM of ethanolamine for 20 min. Finally, 10 nM of biotinylated DNA aptamer was incubated on streptavidin-coated electrode for 40 min, and washed thoroughly with distilled water.

Electrochemical analysis

Various concentration of tetracycline (1 nM–100 μM) in binding buffer (100 mM NaCl, 20 mM Tris–HCl, pH 7.6, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02% Tween 20) was dropped on electrode and incubated for 30 min. After washing with distilled water, 50 μL of 5 mM $K_3Fe(CN)_6$ solution containing 100 mM KCl was dropped on electrode chip until all three electrodes are immersed. Then, CV was performed under potential range of −0.6 to 0.6 V with a scan rate of 20 mV/s and a step potential of 2 mV. The SWV was measured under electric potential range of −0.6 to 0.6 V with a frequency of 8 Hz, step potential of 5 mV, and amplitude of 2 mV. To verify that the current change is caused by only specific interaction between TET and anti-TET aptamer; the electrochemical analysis for 10 μM of oxytetracycline, doxycycline, and diclofenac was evaluated under the identical condition. Figure 2b shows a schematic diagram of signal off system by interfering in the electron transfer.

The electrochemical data analysis was carried out, and the decreasing percent of current before and after the sample treatment ($\Delta I = (I_0 - I_1)/I_0 \times 100$) was measured. Where ΔI is relative current change, I_0 and I_1 represent the current before and after the sample treatment, respectively.

Results and discussion

Immobilization of DNA aptamer on gold electrode

A 76-mer ssDNA aptamer, which can specifically bind to TET, was used as a recognition element in electrochemical aptasensor for TET detection. The aptamer was modified by tagging with penta-T residues followed by biotinylation and thiol-modification at 5′-end that facilitated immobilization on a screen-print electrode without losing its binding properties.

First, an electrochemical analysis was carried out for the immobilization of DNA aptamer on screen-printed gold electrode chip by CV and SWV analysis, because the immobilization of TET binding DNA aptamer on gold electrode surface could be greatly affected to the performance of TET detection. The thiol-modification and avidin–biotin interaction is commonly used methods for the immobilization of DNA on gold surface; therefore, both thiol-modified and biotinylated TET binding DNA aptamer were tested in EC aptasensor system. Our results showed that the biotinylated aptamer immobilized on streptavidin-modified gold electrode showed better sensitivity for TET detection (data not shown). This result is consistent with a previous report that the coverage of the thiol-modified ssDNA was lower than that of biotinylated ssDNA on the gold surface [44]. CV and SWV analysis was conducted at each and every step in order to characterize the immobilization of the aptamer on gold electrode. The change in current occurred in cyclic voltammogram after both the coating of streptavidin as well as aptamer immobilization.

The concentration of TET binding DNA aptamer immobilized on gold electrode was also optimized using a series of aptamer concentrations to determine the effective concentration of ssDNA aptamer to be immobilized for further studies. The optimal concentration of TET binding ssDNA aptamer was determined to be 10 nM at which the current changes by TET binding to aptamer was most effective in both of CV and SWV analysis (data not shown). Too low concentration of aptamer may not yield an efficient electrochemical signal, while the dense immobilization of aptamer may mask the gold electrode surface and result in decrease in sensitivity of the detection.

Quantitative detection of tetracycline

The SWV analysis was conducted against a series of TET concentrations ranging from 1 nM to 100 μM on electrode

surface immobilized with 10 nM ssDNA aptamers. The concentration-dependent decrease in current was observed in CV and SWV (Fig. 3a, b). Figure 3a shows the current of only aptamer-immobilized gold electrode before the TET treatment. The current was found to be slightly decreased with 1–10 nM TET treatment, and this was taken as a background signal because addition of buffer also showed similar level of current drop. However, the current was decreased considerably upon treatment with TET from a concentration above 100 nM even if the linearity between 10 nM and 10 μM was not significantly high. The current change was found to be saturated above 10 μM TET concentrations. The binding of TET to aptamer immobilized on the gold electrode may have affected the rate of electron flow produced from a redox reaction between ferrocyanide and ferricyanide. Therefore, the decrease in current after TET treatment was based on the specific interaction between aptamer and TET that influenced the change in current. Studies on the interaction between aptamers and small molecules indicated that low-molecular targets are often pocketed into the folded aptamer structure, and it is difficult to perform a sandwich assay. But a simple single-site binding assay is generally limited for the development of a sensitive EC aptasensor for small molecules because the electron transfer resistance or current changes by the binding of small molecular targets is usually low as compared to macromolecules like proteins. In previous reports, the binding of macromolecule like thrombin to aptamer brings decrease in current due to interference in the electron flow statically. For small chemical, however, the interfering effect by binding is probably not as high as with proteins. The exact mechanism of current change by the binding of small chemical to its aptamer is still unclear. However, it is assumed that the formation of aptamer–TET complex probably changes the permeability of the layer toward charged ferricyanide ions, and hence the rate of their diffusion due to the electrical property of TET and the conformational change of aptamer. It is well documented that tetracycline molecules are charged and contain three ionisable groups (tricarbonyl, dimethylammonium, and phenolic-diacetone), the pK values of which are 3.3, 7.8, and 9.6 in aqueous solutions, respectively [45]. In addition, the fact that aptamers also change their conformational structure once they bind to their target molecule and contribute to the current change. In this study, therefore, TET induced folding of aptamer through formation of a stem-loop structure, which probably have induced the change in electrostatic property. Therefore, these changes may have affected the diffusion of redox-mediator and thus change in the current, which was measured by CV and SWV [46, 47].

A linear relationship between the logarithmic TET concentration and current change (ΔI) was plotted

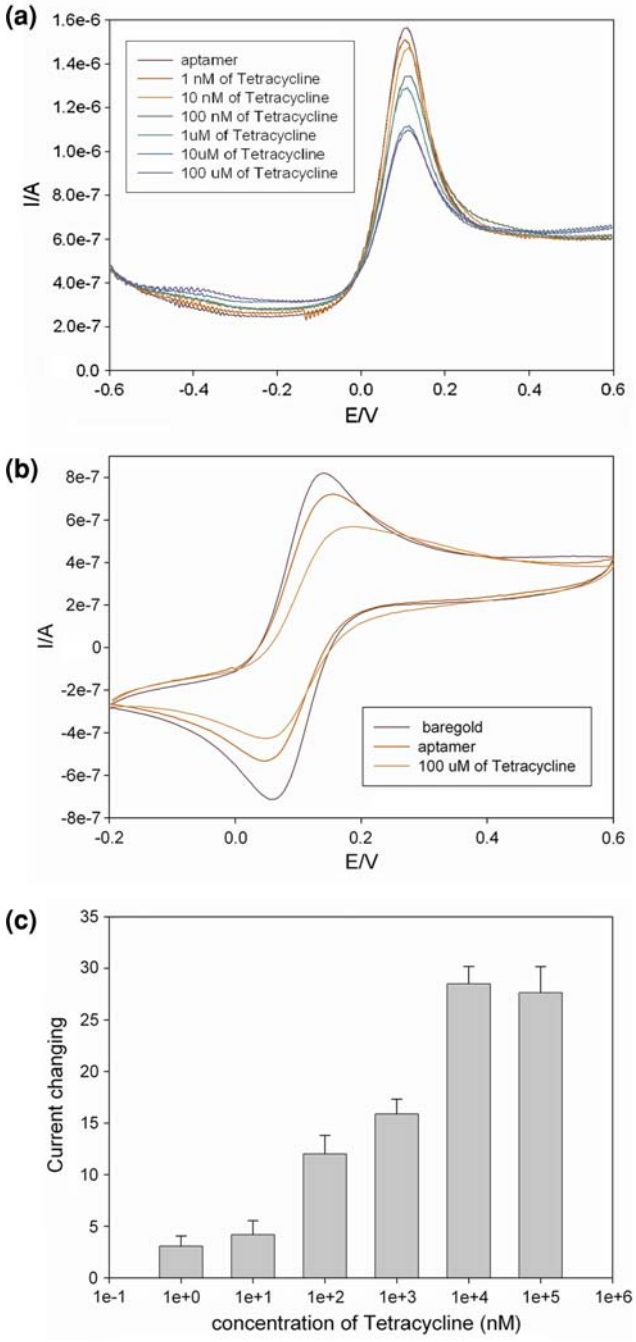

Fig. 3 Electrochemical analysis of TET using modified TET-aptamer-immobilized screen-printed electrode chip: **a** considerable current drop was occurred by the treatment of TET in a range of 100 nM–100 μM from square wave voltammogram, **b** cyclic voltammogram showing current changes after immobilization of aptamer and treatment of maximum concentration of tetracycline, and **c** current drop ratio by increasing tetracycline concentrations with a linear detection range from 10 nM–10 μM of TET

($R^2 = 0.955$), and dynamic range was determined to be in the range 10 nM–10 μM of TET (Fig. 3c). The limit of detection was about 10 nM TET which is similar to the value reported using HPLC detection method [10].

No current decease was detected when 100 μM of tetracycline was treated on the 10 nM aptamer immobilized on the electrode chip possibly because of the saturation. However, the sensitivity for higher levels of TET detection can be improved by expanding the area of the chip and concentration of aptamer immobilized on the electrode chip. In addition, there was a baseline current change detected to be lower than 4%, and this level was also seen when the bare electrode chip (without any aptamer immobilized) was treated with 10 μM tetracycline (current drop = 3.9%).

Specificity of EC aptasensor for TET

Three structurally similar tetracycline derivates, such as tetracycline (TET), doxycycline (DOX), and oxytetracycline (OTC), and a structurally distinct molecule diclofenac was chosen (Fig. 4). Specificity test was conducted similar to that of the sensitivity tests. Figure 5a shows the current changes with a maximum concentration (10 μM). The results showed that the current after oxytetracycline (OTC) and doxycycline (DOX) treatment was slightly decreased to about 10 and 8%, respectively. This value corresponds to the current change when 50 nM of TET was treated (Fig. 5a). It was assumed that the anti-TET aptamer used in this study can also weakly bind to OTC and DOX reported in previous studies [42]. The binding of this anti-TET aptamer for the other antibiotics in tetracycline group was because of the initial steps employed during the selection of TET aptamer. It was reported that the selection of TET aptamer initially began with the OTC as the primary target molecule followed by screening for those which have high specificity and selectivity to TET as the target molecule [42]. Therefore, the aptamer used in this study undoubtedly have specificity and selectivity to TET (Fig. 5a). However,

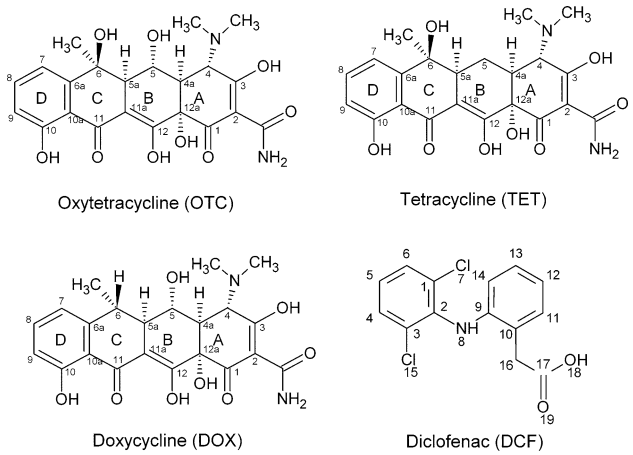

Fig. 4 Chemical structures of tetracycline, oxytetracycline, doxycycline, and diclofenac

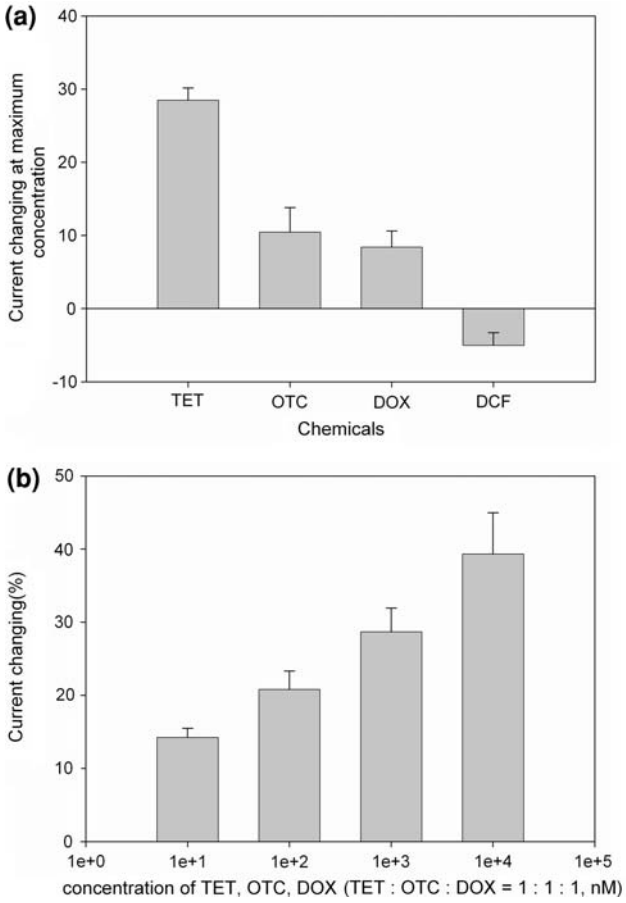

Fig. 5 Specificity test using the tetracycline aptasensor: **a** aptasensor responses to various targets (10 μM). TET, OTC, and DOX are three structurally similar target molecules while, diclofenac (DCF) is a structurally distinct chemical compound used to test the specificity of aptasensor; and **b** selectivity of aptasensor to TET in presence of an equimolar mixture ratio (1:1:1) of three structurally similar tetracycline derivatives (TET, OTC, and DOX) in a series of concentrations ranging from 10 nM to 10 μM. The figure showing current drop proportionality to tetracycline concentration was not affected by the presence of structurally related chemicals

this aptamer can also weakly recognize the other tetracyclines, such as OTC and DOX due to the minor structural changes, differential binding strength and specificity. The current drop against the weak interaction of TET-aptamer with OTC and DOX was below 10% compared to the current drop with TET as target molecule at a same concentration (30%). Further, a structurally distinct chemical, the diclofenac was used to test the specificity of this aptasensor to ascertain that the aptasensor was specific to TET or related compounds. It was found that there was no significant current drop against diclofenac molecule, indicating that the aptasensor was specific to TET with an additional property to distinguish the other TET derivatives.

The electrochemical aptasensor was also tested using a mixture of three tetracycline derivatives (TET, OTC, and DOX) for its specificity (Fig. 5b). These chemicals were

mixed in a series of equimolar concentrations ranging 10 nM–10 μM and analyzed for the specificity of aptasensor. The results showed that the current drop was linearly proportional to the concentrations of the target molecule mixture. The lower limit of detection was 10 nM and the maximum of 10 μM from an equimolar mixture of TET:OTC:DOX. The above result indicated that the binding of TET was not interfered by the presence of structurally similar derivatives at least in equimolar concentrations. The aptasensor developed in this study is unique in that it specifically distinguishes TET with a broad detection limit (10 nM–10 μM).

In a previous report from this laboratory, an electrochemical aptasensor was developed using gold IDA-electrode chip that was highly specific to OTC but failed to detect TET. In addition, the detection limit using aptasensor for OTC had a narrow range of only 1–100 nM OTC [47]. This work is an extension of our previous work where we report on aptasensor specific for TET that also distinguishes TET from other structurally related tetracycline group of antibiotics. The aptasensor based on the screen-print electrode surface functionalized with modified TET-aptamer gave a broader sensitivity with a detection limit from sub-nanomolar to micromolar range. This aptasensor having a broader detection limit may be applicable for detection of TET at a low to high levels in pharmaceutical preparations and contaminated food and waters with high molecular specificity compared to the conventional detection techniques, such as HPLC or GC.

Conclusion

An aptamer-based electrochemical biosensor was developed for detection of TET and its derivatives. The electrochemical aptasensor was fabricated after the modified ssDNA aptamer specific for TET ($K_d = 63.6$ nM) was immobilized on a screen-printed electrode, and the electrochemical analysis was conducted by cyclic voltammetry and square wave voltammetry analysis. Our results showed that the electrochemical aptasensor showed better sensitivity to TET with a detection range of 0.01–10 μM. In addition, the aptasensor also showed selectivity to TET, and this was evidenced after using a mixture of other tetracycline derivatives (OTC and DOX) along with TET. The aptasensor did not show any response to a structurally distinct chemical model, diclofenac, indicating that the aptasensor was specific to TET. Finally, the aptasensor developed in this study can be potentially applied for detection of trace to higher levels of TET contaminations in food products, such as milk, meat, chicken/egg, pharmaceutical preparations, and drinking water.

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